

PCT/NZ2004/000308

REC'D 28 DEC 2004

WIPO PCT

## CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 28 November 2003 with an application for Letters Patent number 529860 made by OVITA LIMITED.

Dated 13 December 2004.

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)



Neville Harris  
Commissioner of Patents, Trade Marks and Designs



529860

Patents Form No. 4

Our Ref: JC218744

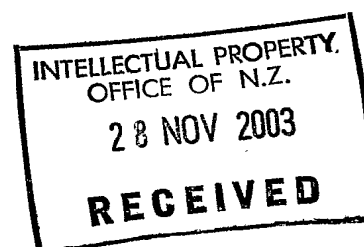
Patents Act 1953

PROVISIONAL SPECIFICATION  
NOVEL MUSCLE GROWTH REGULATOR

We, **OVITA LIMITED**, a New Zealand company, of Level 4, NZI House,  
9 Moray Place, Dunedin New Zealand do hereby declare this invention to be  
described in the following statement:

- 1 -

PT043870589



## NOVEL MUSCLE GROWTH REGULATOR

### Field of the Invention

This invention relates to a novel protein involved in the regulation of muscle growth and the use of the novel protein in regulating muscle growth and treating diseases associated with abnormal muscle growth.

### Background of the Invention

Muscle tissue comprises large, multinuclear cells. The bulk of these cells, approximately two thirds, is myofibrils, or the contractile units. Myofibrils are made up of myosin thick filaments and actin thin filaments.

The development of a muscle cell begins with a myoblast or precursor cell. Myoblasts undergo a differentiation and fusion process to form large myotubes, which in turn differentiate further to become muscle fibers.

The protein myostatin (or Growth Differentiation Factor 8) has been identified as a major factor in regulating muscle growth and development. Myostatin was shown to negatively regulate muscle growth (Kambadur *et. al.* 1997). An 11bp deletion in myostatin has been shown to cause the Belgian Blue (or double-muscling) phenotype in cattle. Belgian Blue cattle have a 20% to 30% increase in muscle mass.

The exact mechanism by which myostatin acts to retard muscle growth is still being elucidated.

During periods of prolonged disuse (e.g. bed rest, space flight), or in cases of muscle wasting diseases (e.g. muscular dystrophy) skeletal muscle undergoes atrophy, which is primarily due to enhanced degradation of muscle protein and a reduction in protein synthesis. Duchenne muscular dystrophy is one of the most common forms of muscular dystrophy. Muscle fibers undergo necrosis and lose their ability to regenerate. It has been shown recently that in mdx mice, a duchenne muscular dystrophy model, muscle is unable to regenerate due to an exhaustion of satellite cells rather than fibrosis. Sarcopenia is the decline in muscle mass and performance associated with normal aging.

The skeletal muscle is still capable of regenerating itself but it appears that the environment in old aged muscle is less supportive towards muscle satellite cell activation, proliferation and differentiation.

5 Many growth factors are involved in regulating postnatal skeletal muscle growth and development for example IGF, HGF and FGF. No known growth factor has a more potent negative effect on skeletal muscle development than myostatin (GDF-8). Myostatin or Growth and Differentiation Factor-8 (GDF-8) was first characterised in mice. Myostatin-null mice displayed drastically increased muscle development and weighed 2 to 3 times  
10 more than wild-type mice. The increase in muscle mass was shown to be due to both muscle hyperplasia and hypertrophy. These data suggest that myostatin has an important role in controlling muscle mass and that myostatin is a potent negative regulator of muscle growth.

15 Therefore, it would be beneficial to identify further factors involved in the regulation of muscle growth, including a factor that is able to promote muscle growth. To date, no further factor has been identified which is able to regulate muscle growth.

#### **Statement of the Invention**

20 The present invention is based upon the identification of a polypeptide involved in promoting muscle growth. This muscle growth promoter has been termed "mighty". The term mighty is used throughout this specification to refer to the novel muscle growth promoter according to the present invention.

25 The present invention is also based on the identification of the DNA that encodes the mighty protein and the corresponding mighty gene promoter.

The present invention provides for an isolated polynucleotide including a sequence selected from SEQ ID No. 1 or SEQ ID No. 3.

30 The present invention also provides a polypeptide encoded by a polynucleotide including a sequence selected from SEQ ID No. 1 or SEQ ID No. 3. The polypeptide may include a sequence selected from SEQ ID No. 2 or SEQ ID No. 4.

35 The present invention also provides one or more vectors comprising the sequences of the present invention, and one or more host cells containing such vectors.

The invention also provides a composition for regulating muscle growth.

In one aspect the composition includes any one of:

- a) a polynucleotide including SEQ ID No. 1 or SEQ ID No.3,
- b) a fragment or variant of (a),
- c) a polynucleotide having 95%, 90% or 70% sequence identity to (a),
- d) a complement of any one of (a) to (c),
- e) a reverse complement of any one of (a) to (c),
- f) an antisense polynucleotide of any one of (a) to (c),
- g) a polypeptide encoded by any one of (a) to (c),
- h) a polypeptide including SEQ ID No. 2 or SEQ ID No. 4,
- i) a fragment or variant of (g) or (h), and
- j) a polypeptide having 95%, 90% or 70% sequence relating to (g) or (h).

In another aspect the composition may include the mighty gene promoter including a sequence of SEQ ID No. 5, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or a fragment or variant thereof.

In a further aspect the composition may include a modulator of mighty gene expression or mighty protein activity.

The modulator of mighty gene expression or mighty protein activity may specifically bind to a polynucleotide selected from any one of:

- a) SEQ ID No.1, SEQ ID No. 3, or SEQ ID No. 5,
- b) a polynucleotide that encodes a polypeptide of SEQ ID No. 2 or SEQ ID No. 4,
- c) a polynucleotide having 95%, 90% or 70% identity to (a) or (b),
- d) a complement of any one of (a) to (c),
- e) a reverse complement of any one of (a) to (c), and
- f) a fragment or variant of any one of (a) to (e).

The modulator of mighty gene expression may be an anti-sense polynucleotide. The modulator of mighty gene expression may also be an interfering RNA molecule. Specifically, the modulator of mighty gene expression may be an RNAi or siRNA molecule.

In a further aspect, the compositions of the present invention may be used in the treatment or prophylaxis of diseases associated with muscle growth. The disease may be a disease that results in muscle atrophy. The disease may be selected from muscular dystrophy, muscle cachexia, atrophy, hypertrophy or diseases associated with cardiac muscle growth, including infarct. The composition may also be used in promoting muscle regeneration after muscle injury.

The present invention also provides for a method of regulating muscle growth using a composition according to the present invention. The method may be used to produce an animal having increased muscle mass.

The invention also provides for a transgenic animal transfected with a composition according to the present invention. The transgenic animal may result in an animal having an increased muscle mass. The transgenic animal may be selected from a sheep, cow, bull, deer, poultry, turkey, pig, horse, mouse, rat or human.

The present invention also provides a method of predicting muscle mass in an animal, including the steps of:

- i) obtaining a sample from the animal,
- ii) determining the gene expression level from a polynucleotide having a sequence of SEQ ID No.1 or SEQ ID No.3, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 1 or SEQ ID No.3, or a fragment or variant thereof; or determining the amount of a polypeptide having a sequence of SEQ ID No.2 or SEQ ID No.4, a polypeptide having 95%, 90% or 70% identity to SEQ ID No. 2 or SEQ ID No.4, or a fragment or variant thereof,
- iii) comparing the gene expression level or amount of polypeptide to an average; and
- iv) predicting the muscle mass of said animal.

The level of gene expression may be determined using RTPCR or northern analysis. The polypeptide may be determined using ELISA or Western blot analysis.

The invention also provides a method of improving the muscle mass of an animal comprising the steps of:

- i) selecting one or more animals predicted to have an increase in muscle mass

according to the present invention, and

- ii) breeding the one or more animals predicted to have an increased muscle mass to produce an animal having an improved muscle mass.

5 The animal according to the present invention may be selected from a sheep, cow, bull, deer, poultry, turkey, pig, horse, mouse, rat or human.

The invention also provides for antibodies that preferentially bind a polypeptide having a sequence of SEQ ID NO. 2 or SEQ ID NO. 4 or a polypeptide having 95%, 90% or 70% identity to SEQ ID NO. 2 or SEQ ID NO. 4.

The invention also provides for the use of an antigenic fragment of a polypeptide having a sequence of SEQ ID NO. 2 or SEQ ID NO. 4 in the production of an antibody that preferentially binds a sequence of SEQ ID NO. 2 or SEQ ID NO. 4 or a polypeptide having 95%, 90% or 70% identity to SEQ ID NO. 2 or SEQ ID NO. 4.

The present invention also provides an isolated polynucleotide comprising a sequence of SEQ ID No: 5, which comprises the promoter region of the murine mighty gene, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or a fragment or variant thereof.

The present invention also provides one or more vectors comprising a polynucleotide of SEQ ID No: 5, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or the fragment or variant thereof, and one or more host cells containing such vectors.

The present invention also provides a method of screening for one or more compounds that are potentially useful in inhibiting or promoting muscle growth, including the steps of:

- i) inserting a polynucleotide having a sequence of SEQ ID No: 5, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or a fragment or variant thereof into a suitable vector linked to a suitable marker gene;
- ii) transforming a suitable host cell with the vector;
- iii) administering a compound of interest to the host cell; and
- iv) determining any difference in the level of the marker gene expression.

35 The vector may include any suitable vector, and may include, a prokaryotic plasmid, a eukaryotic plasmid or a viral vector.

The marker gene may include any suitable marker gene, and may include a polynucleotide that encodes a green fluorescent protein, a red fluorescent protein, a luciferase enzyme, or a  $\beta$ -galactosidase enzyme.

5 The invention also provides a method of expressing a desired protein in a muscle cell, including the steps of:

- i) isolating a polynucleotide sequence that encodes the gene to be expressed;
- ii) inserting a polynucleotide having a sequence of SEQ ID No: 5, or a polynucleotide  
10 having 95%, 90% or 70% identity to SEQ ID No. 5, or a fragment or variant thereof, operably linked to the polynucleotide sequence of the protein to be expressed in a 5' – 3' orientation, into a suitable vector, and
- iii) introducing the vector into a muscle host cell.

15 The vector may include a eukaryotic vector, viral vector, or any vector suitable for gene therapy.

The host cell may include a primary myoblast cell line, a transformed myoblast cell line or any cell line in which the mighty promoter is active. The host cell may also include an *in*  
20 *vivo* skeletal or cardiac muscle cell of a host animal.

The host animal may include a sheep, cow, deer, bull, poultry, turkey, pig, horse, mouse, rat or human.

25 **Definitions:**

The term "polynucleotide" is to be understood as meaning a polymer of deoxyribonucleic acids or ribonucleic acids, and includes both single stranded and double stranded polymers, including DNA, RNA, cDNA, genomic DNA, recombinant DNA and all other  
30 known forms of polynucleotides. The polynucleotide may be isolated from a naturally occurring source, produced using recombinant or molecular biological techniques, or produced synthetically. A polynucleotide may include a whole gene or any part thereof, and does not have to have an open reading frame.

35 The use of all polynucleotides according to the present invention includes any and all open reading frames. Open reading frames can be established using known techniques



in the art. These techniques include the analysis of the sequences to identify known start and stop codons. Many computer software programmes that can perform this function are known in the art.

5 The term "polypeptide" is to be understood as meaning a polymer of covalently linked amino acids. A polypeptide includes a polypeptide that has been isolated from a naturally occurring source, a polypeptide that has been produced using recombinant techniques, or a polypeptide that has been produced synthetically. It is to be appreciated that a polypeptide that includes a leader or pro-sequence which is cleaved off *in vitro*, or a  
10 polypeptide that includes a linker or any other sequence, or a polypeptide that undergoes a post-translational modification is intended to come within the definition of polypeptide.

The term "fragment or variant" is to be understood to mean any partial sequence or sequence that has been modified by substitution, insertion or deletion of one or more  
15 nucleotides or one or more amino acid residues, but has substantially the same activity thereof.

A polynucleotide fragment also includes a polynucleotide fragment of sufficient length and specificity to hybridise under stringent conditions to a sequence of SEQ ID No: 1 of SEQ  
20 ID No: 3. An example of "stringent conditions" involves pre-hybridisation with 5X SSC, 0.2% SDS at 65°C; performing the hybridisation overnight in 5X SSC, 0.2% SDS at 65°C; two washes of 1X SSC, 0.1% SDS at 65°C for 30 min each; followed by a further two washes of 0.2X SSC, 0.1% SDS at 65°C, also for 30 min each.

25 A polypeptide fragment also includes a fragment that retains the activity of the mighty protein. This fragment may have enhanced activity and therefore, when introduced or expressed in a cell, results in an increase in mighty protein activity. Alternatively, the fragment may have a dominant negative effect.

30 "Mighty gene" is defined as a polynucleotide according to SEQ ID No. 1 or SEQ ID No. 3, or a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 1 or SEQ ID No. 3 or a fragment thereof.

35 "Gene expression" is defined as the initiation of transcription, the transcription of the mighty gene into mRNA, and the translation of the mRNA into a polypeptide. "A modulator of mighty gene expression" is defined as any compound that is able to cause

an increase or a decrease in mighty gene expression.

"Mighty protein" is defined as a polypeptide having a sequence of SEQ ID No. 2 or SEQ ID No. 4, a polypeptide having 95%, 90% or 70% identity to SEQ ID No. 2 or SEQ ID No. 4, or a fragment or variant thereof.

"Mighty protein activity" is defined as the ability of the mighty protein to stimulate muscle growth.

"Muscle growth" is defined as the division and/or differentiation of muscle cells and includes the division and/or differentiation of any muscle precursor cell.

"A modulator of mighty protein activity" is defined as a compound that is able to increase or decrease mighty protein activity.

The "mighty gene promoter" is defined as a polynucleotide of SEQ ID No. 5, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or a fragment or variant thereof.

Further aspects of the present invention will become apparent from the following Figures and description, given by way of example only.

**Brief Description of the Figures:**

The invention will now be described by way of example only with reference to the following figures:

Figure 1: Shows the PCR amplification of mighty from double muscled cattle and normal muscled cattle.

Figure 2: (A) shows the PCR amplification of mighty from the heart tissue of a normal muscled cattle (wt, lane 1) and a double muscled phenotype (BB, lane 2). (B) shows the PCR amplification of mighty from ovine skeletal muscle (lane 4).

Figure 3: (A) and (B) shows the mighty promoter sequence, and the identified transcription factor binding sites.

Figure 4: Shows the results of expression of mighty in myoblast C2C12 cell proliferation.

Figure 5: Shows immunostaining of control and mighty over-expressing myotubes with MHC antibody.

#### **Detailed Description of the Invention:**

In one aspect, the present invention provides for the mighty polynucleotide isolated from bovine and ovine. Specifically, the invention provides a polynucleotide sequence from ovine, SEQ ID No. 1, and bovine SEQ ID No. 3 including anti-sense polynucleotides and operable anti-sense fragments.

The present invention also provides for a polypeptide sequence isolated from bovine and ovine. Specifically, the invention provides a polypeptide from ovine, SEQ ID No. 2 and bovine SEQ ID No. 4.

The invention also provides for vectors containing polynucleotides of the present invention. Vectors are intended to include the incorporation of a sequence according to the present invention into a plasmid and/or virus to aid in the introduction and/or maintenance of the sequence in a host cell. The host cell may include, either, a prokaryotic or a eukaryotic cell. The eukaryotic cell may be *in vivo*, or may be a primary or transformed cell line.

The mighty protein has been shown to be highly expressed in doubled muscled cattle (see figure 1) indicating that mighty plays a role in promoting muscle growth. Mighty has also been shown to up regulate the growth of myoblast C2C12 cells, confirming mighty's role in promoting muscle growth. Therefore, mighty provides a useful tool to regulate muscle growth.

In a further aspect the present invention provides for compositions for regulating muscle growth. The "regulation of muscle growth" is intended to include any change in the rate of muscle growth and/or development and includes the growth and/or differentiation of any

muscle precursor cell. This includes any change in rate at which precursor muscle cells divide, and/or any change in the rate at which precursor muscle cells differentiate. The change may be either an increase or a decrease.

5 In one aspect the composition may comprise a mighty gene sequence from ovine SEQ ID No. 1, or bovine SEQ ID No. 3, a polypeptide having 95%, 90% or 75% identity to SEQ ID No. 1 or SEQ ID No. 5, or a fragment or variant thereof. The sequence may be introduced into a cell by incorporation into a suitable vector under the regulation of a promoter, either the mighty promoter (SEQ ID No: 5), or any other suitable promoter. The promoter may  
10 be used to cause expression of the mighty protein, thereby both increasing gene expression and mighty protein activity within the cell.

The composition may also include a sequence having 95%, 90% or 70% identity to the polynucleotide sequences of the present invention. Sequence identity may be determined  
15 by aligning the sequences and determining the number of identical nucleotides. Many computer algorithms are known for determining sequence identity, for example, the BLASTN algorithm.

The composition may also include complements, reverse complements, or anti-sense polynucleotides of the polynucleotides according to the present invention.  
20

The composition may also comprise a mighty polypeptide according to the present invention. The polypeptide may be from ovine, SEQ ID No. 2, or bovine, SEQ ID No. 4, polypeptides having 95%, 90% or 70% identity to SEQ ID No. 2 or SEQ ID No. 4, or  
25 fragments or variants thereof.

The composition may also comprise a sequence having 95%, 90% or 70% identity to the polypeptide sequences of the present invention. Sequence identity may be determined by aligning the sequences and determining the number of identical residues. Many computer  
30 algorithms are known in the art for determining the sequence identity, for example BLASTP algorithm.

The composition for regulating muscle growth may also comprise a modulator of mighty gene expression.  
35

The composition for regulating muscle growth may also include a modulator of mighty protein activity.

5 The modulator of mighty gene expression may be a compound that can specifically bind to a polynucleotide according to the present invention. Specifically, the modulator of mighty gene expression could bind to the mighty gene promoter, thereby affecting the rate at which gene transcription is initiated. Alternatively, the promoter or a fragment thereof could be used to introduce specific alterations into the native promoter of a cell to either enhance or repress wild type mighty gene expression. Alterations can include  
10 substitutions, inserts, or deletions of one or more nucleotides.

The modulator of mighty gene expression may also bind to the mighty gene directly affecting the rate at which the gene is expressed.

15 The modulator of mighty gene expression may also bind to the mighty gene and introduce alterations into the sequence, for example, by homologous recombination, which may either affect the rate at which the gene is expressed, or may alter the mighty protein activity. Alterations of a sequence include a nucleotide change, insertion or deletion, which may or may not result in an amino acid change, insertion or deletion in the resulting  
20 polypeptide. Examples of alterations can include the insertion of termination codons such that a truncated polypeptide is produced, or the alteration of one or more codons such that one or more amino acid residues are altered. Alternatively, the variations could be to delete a section of the wild type mighty gene, or introduce a section into the mighty wild type gene. Techniques are well known in the art to make such alterations. Furthermore,  
25 it would be within the skill of a person skilled in the art to introduce such changes into the mighty gene and then test the alterations on mighty activity, for example using the myoblast proliferation assay as described in example 4.

30 The mighty gene expression may also be altered by introducing polynucleotides that interfere with transcription and/or translation. For example anti-sense polynucleotides could be introduced, which may include; an anti-sense expression vector, anti-sense oligodeoxyribonucleotides, anti-sense phosphorothioate oligodeoxyribonucleotides, anti-sense oligoribonucleotides, anti-sense phosphorothioate oligonucleotides, or any other means that is known in the art, which includes the use of chemical modifications to  
35 enhance the efficiency of anti-sense polynucleotides.

It will be appreciated that any anti-sense polypeptide need not be 100% complementary to the polynucleotides in question, but only needs to have sufficient identity to allow the anti-sense polynucleotide to bind to the gene, or mRNA to disrupt gene expression, without substantially disrupting the expression of other genes. It will also be understood that polynucleotides that are complementary to the gene, including 5' untranslated regions may also be used to disrupt translation of the mighty protein. Likewise, these complementary polynucleotides need not have 100% complementary, but be sufficient to bind the mRNA and disrupt translation, without substantially disrupting the translation of other genes.

The modulation of gene expression may also comprise the use of an interfering RNA molecule as is known in the art, and include RNA interference (RNAi) and small interfering RNA (siRNA).

Modulation of gene expression may also be achieved by the use of catalytic RNA molecules or ribozymes. It is known in the art that such ribozymes can be designed to pair with a specifically targeted RNA molecule. The ribozymes bind to and cleave the targeted RNA.

Any other technique known in the art of regulating gene expression can also be used to regulate mighty gene expression.

The composition may also include a modulator of mighty activity. A modulator of mighty may include a dominant negative mutant of the mighty protein. A dominant negative effect arises where a mutant acts to block the physiological activity of a wild type protein. This may occur by the dominant negative protein binding to, but not activating, a receptor, while also preventing the wild type protein from binding. Alternatively the dominant negative may act by binding directly to, and inactivating, the wild type protein. Thus the polynucleotides of the present invention can be used to make suitable compositions, or be used to design suitable compositions that regulate the mighty gene expression, and thereby regulate muscle growth. Such techniques could be used to regulate mighty gene expression within a cell, for example within a primary or transformed cell line, or to regulate muscle growth within an animal.

One possible application of the compositions of the present invention is to promote or inhibit muscle cell growth and/or differentiation. The muscle cell can be either a primary

or transformed cell line, or the cell can be an *in vivo* cell of a host animal. Suitable host animals may include sheep, cows, bulls, deer, poultry, pigs, fish, horses, mice, rats or humans.

5 The compositions of the present invention may also be used for the treatment of diseases associated with muscle tissue. Such diseases or injury may include muscular dystrophy, muscle ataxia, or diseases associated with cardiac muscle growth. Similarly the compositions may also be used to promote muscle regeneration after muscle injury.

10 Similarly the compositions could be used to produce transgenic animals. The compositions could be used to produce transgenic animals having an increase in muscle mass. Suitable animals may include sheep, cows, bulls, deer, poultry, pigs, fish, horses, mice, rats or humans. Many techniques are known in the art for producing transgenic animals, and any suitable method could be used.

15 Another application of the present invention may be to predict the muscle mass of an animal. To do this a sample is obtained from an animal. The sample is then analysed for the level of mighty gene expression, or mighty protein. Many techniques are known in the art for measuring gene expression or protein amount. For example, gene expression can  
20 be analysed using quantitative RTPCR or northern analysis. Protein content can be determined using ELISA [Enzyme-linked Immunosorbant Assay] or Western blot analysis.

25 The level of mighty gene expression, or amount of the mighty protein, is then compared to an average. An average level of mighty gene expression is the average level obtained from a sample of animals of average muscle mass. Similarly, the average amount of mighty protein is the amount of protein observed in a sample of animals of average muscle mass.

30 An increased level of mighty gene expression or mighty protein, compared to the average, means that the muscle mass of the animal will be predicted to have an above average muscle mass. A decreased level of mighty gene expression or mighty protein, compared to the average, means that the muscle mass will be predicted to be less than average.

35 The method may be used to pick animals to be involved in a breeding programme to produce offspring with increased, or decreased muscle mass.

The invention also provides for antibodies against the mighty protein. Given the sequences disclosed in the present specification, a person skilled in the art would be able to produce antibodies against the mighty protein. Examples of how antibodies can be produced including the production of hybridoma cells can be found in *Eryl Liddell and Cryer* or *Javois*.

The present invention also provides one or more polynucleotides comprising the mouse mighty promoter. The mighty promoter is a polynucleotide of SEQ ID No: 5, a polynucleotide having 95%, 90% or 70% identity to SEQ ID No. 5, or fragments or derivatives thereof.

The invention also provides for vectors containing the mighty gene promoter. Vectors are intended to include the incorporation of the polynucleotide into a plasmid and/or virus to aid the introduction and/or maintenance of the polynucleotide in a host cell. The host cell may be a prokaryotic cell or a eukaryotic cell, *in vivo* or a primary or transformed cell line.

The mighty gene promoter can be used to screen for compounds that may be useful in regulating mighty gene expression, and therefore could be useful in regulating muscle growth. To do this, the mighty promoter can be placed into a suitable expression vector with a suitable marker gene. A "marker gene" is a gene whose expression product may be identified and quantified. Many suitable marker genes are known and may include, for example, green fluorescent protein, red fluorescent protein, luciferase, or  $\beta$ -galactosidase.

The vector is then placed into a suitable host cell using known transfection techniques. A suitable host cell comprises a cell in which the mighty gene promoter is activated, causing the marker gene to be expressed, and levels of the marker gene expression product can be detected. The compound of interest is then applied to the host cell, and any changes in the marker gene determined.

An increase in the amount of the marker gene expression product compared to the base line indicates that the compound may be enhancing gene expression via the mighty gene promoter and therefore may be useful in promoting muscle growth. A decrease in the amount of the marker gene product compared to the base line indicates the compound may be inhibiting gene expression via the mighty gene promoter and therefore may be useful in inhibiting muscle growth.



The mighty gene promoter may also be used to express a designated gene in a muscle cell. To achieve this, the mighty gene promoter is inserted into a suitable vector in conjunction with the gene of interest. Many suitable vectors are known in the art and may include eukaryotic vectors, viral vectors or any vector suitable for gene therapy.

The vector can then be introduced into a suitable host cell using known transfection techniques.

A suitable host cell can be any muscle cell or mammalian cell where the mighty promoter is activated. The host cell may include, for example, a primary or myoblast cell line, or a transformed myoblast cell line, or a skeletal or cardiac muscle cell of a host animal.

Any host animal where the mighty promoter is active may be used, but may include for example sheep, cows, bulls, deer, poultry, turkey, pigs, fish, horses, mice, rats or humans.

#### **Example 1: Isolation of Mighty cDNA**

**RNA Purification:** RNA was purified from ovine and bovine skeletal muscle and heart tissue samples using TRIZOL (Invitrogen) according to the manufacturer's protocol.

**Amplification of the mighty cDNA:** Amplification of mighty cDNA was carried out in a combined Reverse transcription PCR. First strand cDNA was synthesized in a 20 µl reverse transcription reaction mixture from 5 µg of total RNA, using a Superscript preamplification kit (Invitrogen) according to the manufacturer's instructions. The PCR conditions and the specific primers used for the amplification are as follows:

Amplification of sheep and cattle skeletal muscle mighty cDNA and bovine heart mighty cDNA was performed using the primers:

Forward primer 5' CACCATGGCGTGCGGGGCGCACTG 3' (SEQ ID No. 6)

Reverse primer 5' GGATACATAGCTTGTGTCCT 3' (SEQ ID No. 7)

The PCR was carried out in the presence of Q solution (Qiagen) with initial denaturation at 94°C for 1 min. Subsequently 35 cycles were performed of the following steps, 94°C for 15 s, 60°C for 45 s, 72°C for 1 min, and 1 cycle of final extension at 72°C for 5 min.

PCR amplification of mighty fragment from Belgian Blue and normal muscled cattle (Fig 1).

Primers used:

bcoo3291 Fwd 5'TGAAGCGGCCCATGGAGTTC 3' (SEQ ID No. 8)

bcoo3291 Rev2 5'GGTGGGCTGGTCCTTCTTCATC 3' (SEQ ID No. 9)

The PCR was performed in the presence of Q solution (Qiagen) and Taq polymerase with initial denaturation at 94°C for 1 s followed by 35 cycles of 94°C for 15 s, 62°C for 30 s, 72°C for 45 s and 1 cycle of final extension at 72°C for 5 min.

The PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized. The results in figure 1 show that mighty is over expressed in the double muscled cattle compared to normal muscled animals. This result shows that mighty has a role in promoting muscle growth. Part A, of figure 2, shows that mighty gene expression is also up regulated in the heart tissue of the double muscle animals indicating that mighty is also able to regulate cardiac muscle growth and development as well as skeletal muscle growth and development. Part B of figure 2 shows the presence of mighty expression in ovine skeletal muscle.

Purification of the PCR products: The PCR reactions were run on 0.8% low melting point agarose gel and the gel containing the desired band was cut out. The DNA from the gel was purified using the Wizard PCR preps DNA purification system (Promega).

### **Example 2: Cloning of Mighty cDNA**

The purified cDNA was ligated in to pGEM-T easy vector according to the manufacturer's protocol (Promega). The ligation reaction was transformed into competent E.coli DH 5 alpha bacteria (Invitrogen) according to the manufacturer's protocol. The transformed bacteria were plated on Lennox L broth (LB) agar plates containing ampicillin (50 mg/litre), IPTG and X-gal. The white colonies were seeded in LB plus ampicillin media and the cultures grown overnight. The plasmid DNA was purified from the cultures using Qiagen mini plasmid kit (Qiagen). The plasmid DNA was digested with the restriction enzyme EcoRI, and analysed on an agarose gel. The positive clones were identified by the presence of the right size fragments. The positive clones were sent for sequencing for further confirmation. The ovine mighty polynucleotide sequence is provided in SEQ ID No. 1, and the corresponding polypeptide sequence is provided in SEQ ID No. 2. The

bovine mighty polynucleotide sequence is provided in SEQ ID No. 3, and the corresponding polypeptide sequence as SEQ ID No. 4.

### **Example 3: Generation of Murine Mighty Stable Cell lines**

The ORF of murine mighty was PCR amplified with the following primers:

Fwd 5' CACCATGGCGTGCGGGGCGACACTG 3' (SEQ ID No. 6)

Rev 5' GGATACATAGCTTGTGGCCT 3' (SEQ ID No. 7)

The Pwo polymerase (Roche), Q solution (Qiagen), and mouse est clone (Resgene) as the template, were used for the PCR reaction according to the manufacturer's recommendations. The PCR conditions were as follows: 35 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 1min and one cycle of 72°C for 5 min.

The cDNA of the mouse mighty gene was purified through the Wizard PCR preps DNA purification system (Promega) and was cloned into the TOPO site of the pcDNA3.1DV5HisTOPO vector (Invitrogen) as per manufacturer's protocol. The recombinants were analysed by restriction digestion and the positive recombinant was sequenced.

For the stable transfection of C2C12 myoblasts with the mouse mighty construct, C2C12 myoblasts ( $1 \times 10^7$ ) were washed twice in ice cold 1x HBS (140 mM NaCl, 0.77mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM Hepes (7.1)) and resuspended in 0.5ml ice cold 1xHBS and transferred to a precooled cuvette gap 0.4 cm (BioRad). 10 µg of linearised plasmid DNA was added (linearised with Sca I). After 5 minutes on ice, cells were mixed by agitation and the cuvette was pulsed at 0.24 kV at 960 uF capacitance with resistance set at 200Ω, and the time constant was an average of 36 ms. Cells were incubated for 10 minutes on ice and transferred to 10 ml of DMEM 10%FBS on a 10 cm dish and triturated up and down to break up cellular debris. Cells were then selected with geneticin and individual clones selected. Clones expressing the transgene were identified by Western blot for the V5 tag in the plasmid.

### **Example 4: Myoblast Proliferation Assay**

Prior to assay C2C12 cells (Yaffe and Saxel) and transfected C2C12 clones were grown in D-MEM media (Life Technologies, Grand Island, NY, USA), buffered with NaHCO<sub>3</sub> (41.9 mmol/l, Sigma Cell Culture Ltd, St Louis, MO, USA) and gaseous CO<sub>2</sub>. Phenol red

(7.22 nmol/l, Sigma) was used as a pH indicator. Penicillin ( $1 \times 10^5$  IU/l) and Streptomycin (100 mg/l, Sigma) were routinely added to media, as was 10% foetal bovine serum (Life Technologies Ltd).

Cell proliferation assays were conducted in uncoated 96-well Nunc microtitre plates. C2C12 cultures were seeded at  $3 \times 10^3$  cells/cm<sup>2</sup> in proliferation media. After a 24 hour attachment period media was decanted and fresh proliferation media added back to the plates.

Plates were then incubated in an atmosphere of 37°C and 5% CO<sub>2</sub>. A test plate was fixed at 0, 24, 48 and 72 hours post media change, and assayed for proliferation by the method of Oliver et al. (1989). Briefly, growth media was decanted and cells washed once with PBS then fixed for 30 min in 10% formol saline. The fixed cells were then stained for 30 min with 10 g/l methylene blue in 0.01 M borate buffer (pH 8.5). Excess stain was removed by four sequential washes in borate buffer. Methylene blue was then eluted off the fixed cells by the addition of 100 ml of 1:1 (v/v) ethanol and 0.1 M HCl. The plates were then gently shaken and absorbance at 655 nm measured for each well by a microplate photometer (BioRad model 3550 microplate reader, BioRad, Hercules, CA, USA).

The results in Figure 4 show that the cells transfected with the mighty gene had a higher absorbance indicating a faster rate of growth compared to normal C2C12 cells. This results show that mighty acts to up regulate the growth of myoblast cells.

#### **Example 5: Cloning of the Murine Mighty Promoter**

The 2.1 kb of 5' upstream sequence was amplified using the mouse genomic DNA and the following primers:

Rev 5' AGA TCT GAT CCA ACT CTT CAG CTA C 3' (SEQ ID No. 10)

Fwd 5' GCT AGC CCA CAT TCA CTG TGC AAG 3' (SEQ ID No. 11)

The PCR was carried out using Q solution (Qiagen) and Expand long DNA polymerase (Roche) according to the manufacturer's protocol. The PCR conditions were, 35 cycles of 95°C for 15 s, 52°C for 30 s, 68°C for 3min and one cycle of final extension at 68°C for 7 min.

The PCR product was analysed on a 0.8% agarose gel and purified through a Wizard purification column (Promega). The purified DNA fragment was cloned into the pGEM-T easy vector as mentioned above. The positive recombinants were selected and analysed by restriction digestion and sequencing. The 2.1 kb fragment was cut out from the pGEM-T easy vector by BglII and NheI enzymes for cloning into a luciferase reporter vector pGL3B. The pGL3B was digested with BglII and NheI and the 2.1 kb mighty promoter fragment was ligated to it using T4 ligase. The E.coli DH 5 alpha were transformed with the ligation reaction and plated on LB agar plus ampicillin plates. The cultures were grown in LB plus ampicillin media and plasmid DNA purified as mentioned previously. The plasmid DNA was analysed by restriction digestion and the positive recombinants identified. The positive recombinant (2.1 construct) was confirmed by sequencing (SEQ ID No. 5). For transfection experiments, the DNA was purified using Qiagen Maxi Prep Kit (Qiagen).

The mighty promoter sequence is shown in SEQ ID No. 5. The mighty promoter sequence was also analysed for known transcription factor binding sites (figure 3). These sites show crucial parts of the promoter sequence.

#### **Example 6: Transfection of C2C12 cells**

C2C12 cells were grown in DMEM +10% FBS according to the established protocol (Spiller et al, 2002). The cells were transfected with 2 µg of the 2.1 mouse mighty promoter construct and 1µg of pCH110 (SV40 β-galactosidase control vector, Amersham) using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). 24 hours later the media was changed to DMEM+10%FBS + 0, 2, 4, 6, 8 or 10 µg/ml recombinant myostatin. 24 hours after the media change the cell extracts were made and luciferase assays (Promega) were performed according to the established protocols (Spiller et al, 2002). The assays for beta-galactosidase activity were done according to the protocol (Promega). Luciferase activity was normalised to beta-galactosidase activity.

The cells were incubated in increasing amounts of myostatin. As shown in figure 5, the addition of myostatin resulted in a repression of the luciferase activity. These results imply that myostatin acts to regulate gene mighty expression through initiation of mighty gene transcription at the promoter. These results also further support the role of mighty as a promoter of muscle cell growth and differentiation.

To assess the function of Mighty in promoting myogenesis, the myoblasts stably expressing mighty were allowed to differentiate in low serum media. Immunostaining of MHC was performed to assess the morphology of the myotubes.

5 Mighty over-expressing cell and the parent cell line C2C12s, differentiated for 72 hours in DMEM 2% horse serum, were washed once in PBS then fixed with 70% ethanol:formaldehyde:glacial acetic acid (20:2:1) for 30 seconds, and then rinsed three times with PBS. Cells were then blocked overnight at 4°C in TBS containing 1% normal sheep serum (NSS). Cells were incubated with the primary antibody, 1:100 dilution anti MHC, in TBS/1%NSS for 1 hour. Cells were washed (3 × 5 min) with TBST and incubated with the secondary antibody, 1:100 dilution sheep anti-mouse IgG in TBS/1%NSS for 30 minutes. Cells were washed as before and incubated with the tertiary antibody, 1:100 dilution of streptavidin-biotin peroxidase complex (RPN1051, Amersham), in TBS/1%NSS for 30 minutes. Cells were then washed again as before. MHC immunostaining was visualised using 3,3-diaminobenzidine tetrahydrochloride (DAB; Invitrogen) enhanced with 0.0375% CoCl and then counterstained with Gills haematoxylin, mounted and photographed

As shown in figure 5, with increased myonuclei, indicating that mighty also promotes hypertrophy in muscles.

Wherein the foregoing description reference has been made to integers or components having known equivalents and such equivalents are herein incorporated as if individually set forth.

Although the invention has been described by way of example and with reference to possible embodiments thereof, it is to be appreciated that improvement and or modifications may be made thereto without departing from the scope or spirit thereof.

#### References:

- Eryl Liddell, J. and Cryer, A. (1996) A Practical Guide to Monoclonal Antibodies. Wiley.
- Javois, Lorette C. (1999) Immunocytochemical Methods and Protocols. Humana Press.

Kambadur, R., Sharma, M., Smith, T.P.L. and Bass, J.J. (1997) Mutations in myostatin (GDF-8) in doubled muscled Belgian Blue and Piedmontese Cattle. *Genome Res.* **7**. 910 – 916.

5      Michael P. Spiller, Ravi Kambadur, Ferenc Jeanplong, Mark Thomas, Julie K. Martyn,,John J. Bass and Mridula Sharma (2002). Myostatin is a downstream target gene of basic helix loop helix transcription factor, MyoD. *Mol & Cellular Biology* **22**: 7066-7082.

10     Oliver, M. H., Harrison, N. K., Bishop, J. E., Cole, P. J. and Laurent, G. J. (1989). A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *Journal of Cell Science* **92**, 513-518.

15     Yaffe, D. and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.

OVITA LIMITED

20        
By Its Attorneys

BALDWIN SHELSTON WATERS

1/6  
SEQUENCE LISTING

<110> Ovita Limited

<120> Novel Muscle Growth Regulator

<130> JC218744

<160> 11

<170> PatentIn version 3.1

<210> 1

<211> 576

<212> DNA

<213> ovine

<400> 1  
atggcggtgcg gggcgacact gaagcggccc atggagttcg aggcggcgct gctgagccct 60  
ggctctccga agcggcgggcg ctgcgcccct ctgtccggcc ccactccggg cctcaggccc 120  
ccggacgccc aaccgccgcc gctgcttcag acgcagaccc caccgccgac tctgcagcag 180  
ccccccccgc ccggcagcga gcggcgccctt ccaactccgg agcaaatttt tcagaacata 240  
aaacaagaat atagtcgtta tcagaggtgg agacatttag aagttgttct taatcagagt 300  
gaagcttgta cttcgaaag tcagcctcac tcctcagcac tcacagcacc tagttctcca 360  
ggttcctcct ggatgaaaaa ggaccagccc acctttaccc tccgacaagt tggaataata 420  
tgtgagcgtc tcttaaaaga ctatgaagat aaaattcggg aggaatatga gcaaattcctc 480  
aataactaaac tagcagaaca atatgaatct tttgtgaaat tcacacatga tcagattatg 540  
cgacgatatg ggacaaggcc aacaagctat gtatcc 576

<210> 2

<211> 192

<212> PRT

<213> ovine

<400> 2

Met Ala Cys Gly Ala Thr Leu Lys Arg Pro Met Glu Phe Glu Ala Ala



[illegible]

gggtcctcct ggatgaaaaa ggaccagccc acctttacgc tccgacaagt tggaataata 420  
 tgtgagcgtc tcttaaaaga ctatgaagat aaaattcggg aggaatatga gcaaattcctc 480  
 aataactaaac tagcagaaca atatgaatct tttgtgaaat tcacacatga tcagattatg 540  
 cgacgatatg ggacaaggcc aacaagctat gtatcc 576

<210> 4  
 <211> 192  
 <212> PRT  
 <213> Bovine

<400> 4

Met Ala Cys Gly Ala Thr Leu Lys Arg Pro Met Glu Phe Glu Ala Ala  
 1 5 10 15

Leu Leu Ser Pro Gly Ser Pro Lys Arg Arg Arg Cys Ala Pro Leu Ser  
 20 25 30

Gly Pro Thr Pro Gly Leu Arg Pro Pro Asp Ala Glu Pro Pro Pro Leu  
 35 40 45

Leu Gln Thr Gln Ile Pro Pro Pro Thr Leu Gln Gln Pro Ala Pro Pro  
 50 55 60

Gly Ser Asp Arg Arg Leu Pro Thr Pro Glu Gln Ile Phe Gln Asn Ile  
 65 70 75 80

Lys Gln Glu Tyr Ser Arg Tyr Gln Arg Trp Arg His Leu Glu Val Val  
 85 90 95

Leu Asn Gln Ser Glu Ala Cys Thr Ser Glu Ser Gln Pro His Ser Ser  
 100 105 110

Thr Leu Thr Ala Pro Ser Ser Pro Gly Ser Ser Trp Met Lys Lys Asp  
 115 120 125

Gln Pro Thr Phe Thr Leu Arg Gln Val Gly Ile Ile Cys Glu Arg Leu  
 130 135 140

Leu Lys Asp Tyr Glu Asp Lys Ile Arg Glu Glu Tyr Glu Gln Ile Leu  
 145 150 155 160

Asn Thr Lys Leu Ala Glu Gln Tyr Glu Ser Phe Val Lys Phe Thr His  
 165 170 175

Asp Gln Ile Met Arg Arg Tyr Gly Thr Arg Pro Thr Ser Tyr Val Ser  
 180 185 190

<210> 5  
 <211> 2071  
 <212> DNA  
 <213> mouse

<400> 5  
 ccacattcac tgtgcaagtc gtggggaaat acagatgaat aaaggcttcc ttgttattct 60  
 caaggaatgt atgggttttga agcacagtta gacatatatt caaattacag cttcctcctt 120  
 taaaacacta atattccaag gcacactcaa tgttttaaaag gatcacagag tgactaccaa 180  
 agcacgtagc aaaaccctac taagagaggt gtgttttaaaa tgactacca aggacatac 240  
 ttttcaagtc ttctaactgt tcactttgga tctgtttata ccacaagaaa acaatttact 300  
 tgatgctctt aggtcccctt aaaaaataac catcgtgaag tggcttttca tgtccttggc 360  
 ttttattgaa catagaaaca gccatgcaag cggctttaa ggctttatta catcattggt 420  
 tcctaataaa gtcatgacag tctacctttg gaattaaagt gatacacaaa atgatgggtct 480  
 gtgtcctctg gtgaactggt tccattcaga taacacctat tcatcatgac tatggtttca 540  
 tttttcttta gccttcaaga agctcagaac tgaattttta attcagtcatt ttaccaccaa 600  
 gataattgtg agtttttttt ttttaaaaaa actctaattgt tttatttcta gatttttagtt 660  
 taaaccacgt tacatctata ttgacaataa atgtgctaaa ataaacttaa catgggtaat 720  
 gtgcctaggg aggcttgaat cccaatatgg caaaacaaac agaaaaccag caatttggtta 780  
 tgctgtgctg tcttatatatt tacagaaata aatgtgaaag tatatgacct atgttatgat 840  
 ctttaagag tttgtagaaa cggaagagga cttagagaaa agcaaccaa acgaacagga 900  
 ggagaaggaa gaagaggcgg agaaggagga ggaagattgg agatagtatg cttttattgt 960  
 ctaaccccaa gtgtgttgaa gtactgtgac agccatcttg gcaattagaa atgagtatct 1020  
 aaaatttgga ctgttctaga aaaatctgtt acagagataa tgttaaagcc agattacagg 1080  
 aatcacagcc actaatatac aaataattac agaaaggctt tgaatgtgga ggtgttggtc 1140  
 tgatgactct attgatgtat ttgaaagcac tggagttact cccagggaaa attacaacca 1200  
 gagttcccta aagcagaacc tccctgtttt ctattcattt gctgaatatc aaaagcattt 1260  
 tccagccaac agtacggcag agaattctga ttgacctgag gaagaaccag tctgagttgc 1320  
 caagtcggat gaggaagcca actgccaat cagctatcag gggaagttcc taacaccctg 1380  
 gtatcacttg gttagacagt ttaagccagt gagttttctg gtaggattgt tttttgggtt 1440  
 tttttttttc cttttaatcc ttttttgcgt aacacatatc catttagtga tccgattaat 1500  
 ggccgggtca tctatcccca aaatacattc atttgtaaca cacctcccct tccaattttg 1560  
 cccatgattg cacagggttc gtggattaaa taaagtctat ccttagataa cccggttatg 1620  
 tttgtgaaga tttcctggga ctcaagacaa aatcctttga taacccttta gaatcacctc 1680  
 ttttatcggg cagcgggcca agggaacccg ggtctcccag ggtctctccc atcccccgcc 1740

cccgaggccc ctgccgcgca ggtgcgaaaag acctcccagg cactccggc agagagcgtg 1800  
 aagggggggg ccctgggagg ggcggggggcg ggggtgttgc taggcgacca cgctctccgc 1860  
 ccagaccggc ctacttcttc cgcagggggc gccatgggccc gagcccaggc tcgcgggcct 1920  
 cccggatcgg cccttttccg acttcttccc ctctgccggg cggtaggcgca cgcccgtgac 1980  
 gtcacaggag gcggggccag cgcggctgcc gggtagccgga ggcgccattg gagccggcctt 2040  
 ggcttgggag ccgtagctga agagttggat c 2071

<210> 6

<211> 25

<212> DNA

<213> PCR Primer

<400> 6  
 caccatggcg tgcggggcga cactg 25

<210> 7

<211> 21

<212> DNA

<213> PCR Primer

<400> 7  
 ggatacatag cttgttggcc t 21

<210> 8

<211> 20

<212> DNA

<213> PCR Primer

<400> 8  
 tgaagcggcc catggagttc 20

<210> 9

<211> 22

<212> DNA

<213> PCR Primer

<400> 9  
 ggtgggctgg tccttcttca tc 22

<210> 10

<211> 25

<212> DNA

<213> PCR Primer

<400> 10  
agatctgatc caactcttca gctac

25

<210> 11

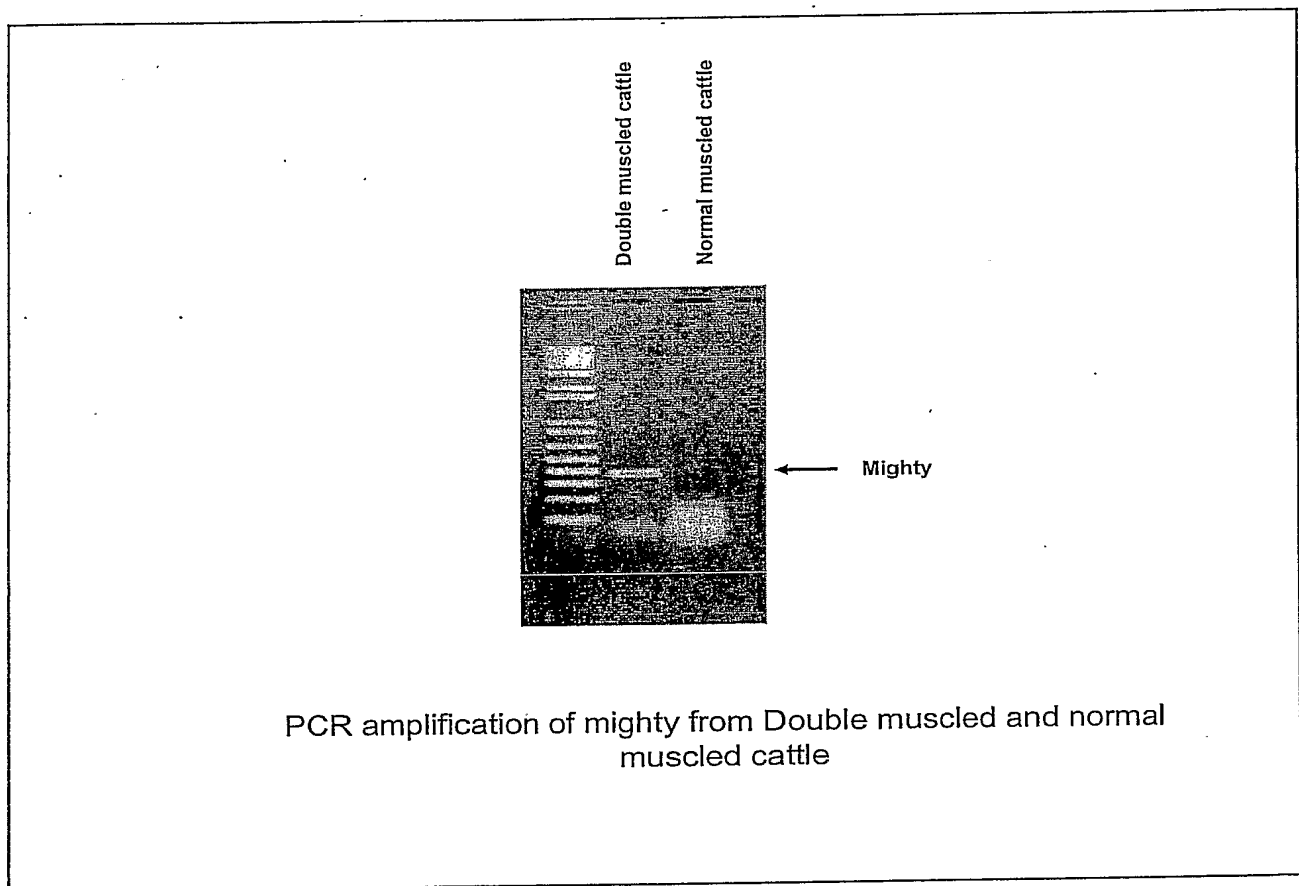
<211> 24

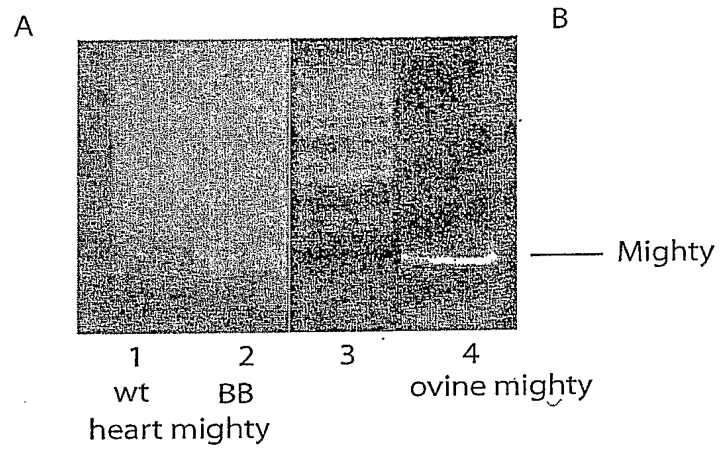
<212> DNA

<213> PCR Primer

<400> 11  
gctagccac attcactgtg caag

24

**FIGURE 1**



Lane 1 PCR amplification of mighty from wild type bovine heart  
Lane 2 PCR amplification of mighty from Belgian Blue bovine heart  
Lane 3 1 kb plus DNA ladder  
Lane 4 PCR amplification of mighty from ovine skeletal muscle

**FIGURE 2**

CCACATTCACCTGTGCAAGTCGTGGGGAAATACAGATGAATAAAGGCTTCCTTGTTATTCTCAAGGAATGTATG  
 GTTTTGAAGCACAGTTAGACATATATTCAAATTACAGCTTCCTCCTTTAAAACACTAATATTCCAAGGCACAC  
 TCAATGTTTTAAAGGATCACAGAGTGACTACCAAGCACGTAGCAAACCCCTACTAAGAGAGGTGTGTTTAAA  
 ATGACTACCCAAGGGACATACTTTTCAAGTCTTCTAATCGTTCACTTTGGATCTGTTTATACCACAAGAAAAC  
 AATTTACTTGATGCTCTTAGGTCCTCTTAAAAAATAGGAAGCTGAAGTGGCTTTTCATGTCCTTGGCTTTTA  
 TTGAACATAGAAACAGCCATGCAAGCGGTCTTAAAGGCTTATTAGATCATTGTTTCTTAATAAAGTCAT  
 GACAGTCTACCTTTGGAATTAAAGTGATACACAAAATGATGGTCTGTGTCCTCTGGTGAAGTGGTTCC  
 TATTATCATTGACTATGGTTTCATTTTTCTTTAGCCTTCAAGAAGCTCAGAACTGAATTTTAAA  
 TTCAGTCATTACCAAGCTTAACTGAGTTTTTTTTTTTTTAAAAAACTCTAATGTTTTATTTCTAGATT  
 TTAGTTTTAAACCACGTTACATCTATATTGACAATAAATGTGCTAAAAATAACTTAACATGGGTAATGTGCCTA  
 GGGAGGCTTGAATCCCAATATGGCAAAACAAACAGAAAACCAGCAATTGGGTATGCTGTGCTGTCTTATATTT  
 TACAGAAAATAAATGTGAAAGTATATGACCTATGTTATGATCTTTAAAGAGTTTGTAGAAACGGAAGAGGACTC  
 AGAGAAAAGCAACCAAAACGAACAGGAGGAGAAGGAAGAAGAGGCGGAGAAGGAGGAG  
 TGCCTTTATTGTCTAACCCCAAGTGTGTTGAAGTACTGTGACGCAATTAGAAATGAGTAT  
 CTAATAATTGGACTGTTCTAGAAAAATCTGTTACAGAGATAATGTTAAAGCCAGATTACAGGAATCACAGCCA  
 CTAATATACAAATAATTACAGAAAGGCTTTGAATGTGGAGGTGTTGTTCTGATGACTCTATGATGTATTTGA  
 AAGCACTGGAGTTACTCCCCAGGAAAATTACAACCAGAGTTCCTAAAGCAGAACCTCCCTGTTTTCTATTCA  
 TTTGCTGAATATCAAAGCATTTTCCAGCCAACAGTACGGCAATTGACCCGAGGAAGAACCAG  
 TCTGAGTTGCCAAGTCGGATGAGGAAGCCAAGTGGGAGTTTCTGGTAGGATTGTTTTTTGGTTTTTTTTTTTCTTTTA  
 TCACCTTGGTTAGACAGTTTAAAGCCAGTGAGTTTTCTGGTAGGATTGTTTTTTGGTTTTTTTTTTTCTTTTA  
 ATCCTTTTTTGCCTAACACATATCCATTTAGTGATCCGATTAATGGCCGGGTCGCTTTTAAATACATT  
 CATTGTAAACACACCTCCCCTTCCAATTTTGCCAGGGTTCGTGGATTAAATAAAGTCTATCC  
 TTAGATAACCCGGTTATGTTTGTGAAGATTTCTGGGACTCAAGACAAAATCCTTTGATAACCCTT  
 TCTTTTATCGGTACGCGGCCAAGGAACCCGGGTCTCCCAGGGTCTCGGATCGCCCCGAGG  
 CCCCTGCCGCGCAGGTGCGAAAGACCTCCCAGGCCACTCCGGCAGAGAGCGTGAAGGGGGGGGCCCTGGGAG  
 GGGCGGGGGCGGGGGTGTGTGCTAGGCGACCAGCTCTCCGCCAGACCGGCCTACTTCTTCCGCAGGGGGCG  
 CCATGGGCCGAGCCCAGGCTCGCGGGCCTCCTTTTCCGACTTCTTCCGCTGCGGGGGGGT  
 GGGCGACGCGCGTGACGTACAGGAGCGGGGGCAGCGGGGCTGCCGGGTGCCGGAGGCGCCATTGGAGCGC  
 GCTTGGCTTGGGAGCGGTAGCTGAAGAGTTGGATC

Various transcription factor binding sites:

Ebox

AP-1

TATA

E4BP4

GC box

E2F

FIGURE 3A



CCACATTCACTGTGCAAGT**CGTGGGG**AAATACAGATGAATAAAGGCTTCCTTGTTATTCTCAAGGAATGTATG  
 GTTTTGAAGCACAGTTAGACATATATTCAAATTACAGCTTCCTCCTTTAAACACTAATATTCCAAGGCACAC  
 TCAATGTTTTAAAGGATCACAGAGTGACTACCAAAGCACGTAGCAAACCCCTACTAAGAGAGGTGTGTTTAAA  
 ATGACTACCCAAGGGACATACTTTCAAGTCTTCTAATCGTTCACCTTTGGATCTGTTTAT**AGGACA**AAGAAA  
 ACAATTTACTTGATGCTCTTAGGTCCCCTTAAAAAATAACCATCGTGAAGTGGCTTTTCATGTCCTTGGCTTT  
 TATTGAACATAGAAACAGCCATGCAAGCGGTCTTAAAGGCTTTATTACATCATTGTTTCCTAATAAAGTCATG  
 ACAGTCTACCTTTGGAATTAAAGTGATACACAAAATGATGGTCTGTGTCCTCTGGTGAAGTGGTTCCATT**CAG**  
**ATAACACCTATT**CATGACTATGGTTTCATTTTTCTTTAGCCTTCAAGAAGCTCAGAACTGAATTTTAAAT  
 TCAGTCATTTACCACCAAGATAATTGTGAGTTTTTTTTTTTTTAAAAAACTCTAATGTTTTATTTCTAGATTT  
 TAGTTTAAACCACGTTACATCTATATTGACAATAAATGTGCTAAAATAAACTTAACATGGGTAATGTGCCTAG  
 GGAGGCTTGAATCCCAATATGGCAAACAAACAGAAAACCAGCAATTTGGTATGCTGTGCTGTCTTATATTTT  
 ACAGAAATAAATGTGAAAGTATATGACCTATGTTATGATCTTTAAAGAGTTTGTAGAAACGGAAGAGGACTCA  
 GAGAAAAGCAACCAAAACGAACAGGAGGAGAAGGAAGAAGAGGCGGAGAAGGAGGAGGAAGATTGGAGATAGT  
 ATGCCTTTTATTGTCTA**AGCCCA**AGTGTGTTGAAGT ACTGTGACAGCCATCTTGGCAATTAGAAATGAGTA  
 TCTAAAATTTGGACTGTTCTAGAAAAATCTGTTACAGAGATAATGTTAAAGCCAGATTACAGGAATCACAGCC  
 ACTAATATACAAATAATTACAGAAAGGCTTTGAATGTGGAGGTGTTGTTCTGATGACTCTATTGATGTATTTG  
 AAAGCACTGGAGTTAC**TCCCCAGG**AAAATTACAACCAGAGTTCCTAAAGCAGAACCTCCCTGTTTTCTATTC  
 ATTTGCTGAATATCAAAGCATTTCAGCCAACAGTACGGCAGAGAATCTCGATTGACCCGAGGAAGAACCA  
 GTCTGAGTTGCCAAGTCGGATGAGGAA**GCCA**ACTGCCAAATCAGCTATC**AGGGGAAGTTCCTA**ACACCCTGG  
 TATCACTTGGTTAGACAGTTTAAAGCCAGTGAGTTTTCTGGTAGGATTGTTTTTTGGTTTTTTTTTTTCCTTT  
 TAATCCTTTTTTTCGTAACACATATCCATTTAGTGATCCGATTAATGGCCGGGTCTCTAT**TCCCCAAA**ATACA  
 TTCATTTGTAACACACCT**TCCCCTT**CCAATTTGCCCATGATTGCACAGGGTTCGTGGATTAAATAAAGTCTAT  
 CCTTAGATAACCCGGTTATGTTTGTGAAGATTTCTGGGACTCAAGACAAAATCCTTTGATAACCCTTTAGAA  
 TCACCTCTTTTATCGGTCACGCGGCCAAGGGAACCCGGGTCTCCCAGGGTCTCTCCCA**TCCCCCGCCCCGAG**  
 GCCCCTGCCG**CGCAGGTGCG**AAAGACCTCCCAGGCCACTCCGGCAGAGAGCGTGAAGGGGGGGGCCCTGGGA  
 GGGGCGGGGGCGGGGGTGTGCTAGGCGACCACGCTCTCCGCCAGACCGGCCTACTTCTTCCGCAGGGGGCG  
 CCATGGGCGGAGCCAGGCTCGCGGGCCTCCCGGATCGGCCCTTTTCCGACT**TTCTTCCCCCTCTGCGGGCGGGT**  
**GGCGCAGCCCGTGACGTACAGGAGCGGGGGCCAGCGCGGCTGCCGGGTGCCGGAGGCGCCATTGGAGCCGG**  
**CTTGGCTTGGGAGCCGTAGCTGAAGAGTTGGATC**

Various transcription factor binding sites:

**MZF1**

**AML-1A**

**Sp1**

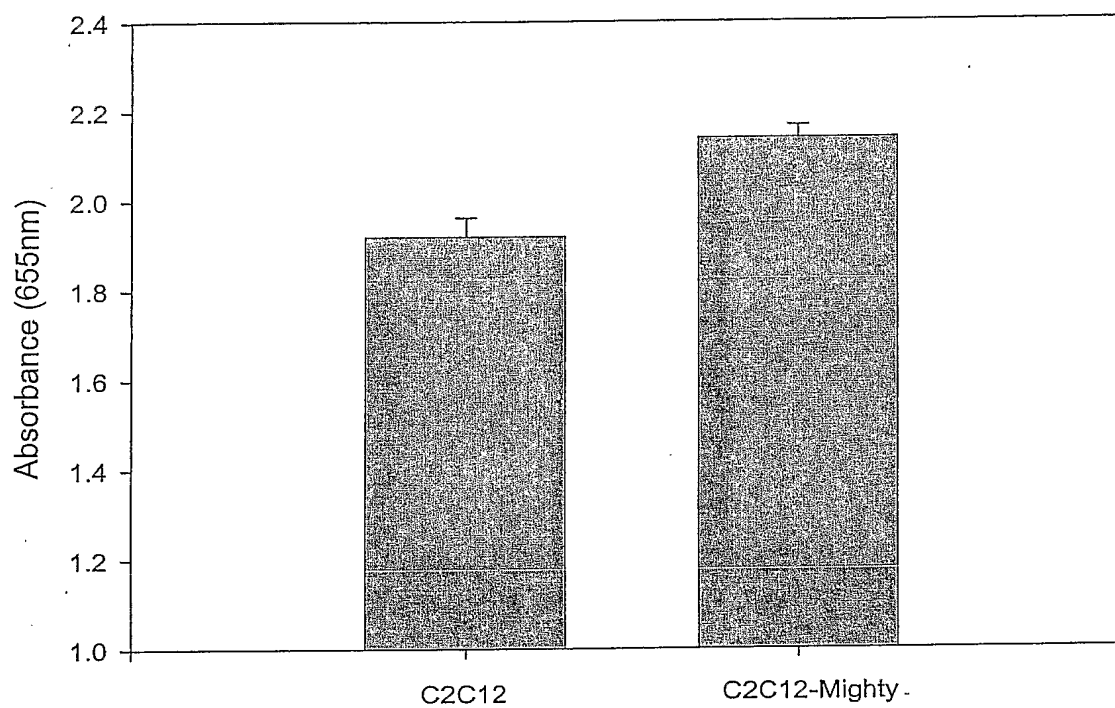
**E47**

**MyoD**

**NF-kap**

**FIGURE 3B**

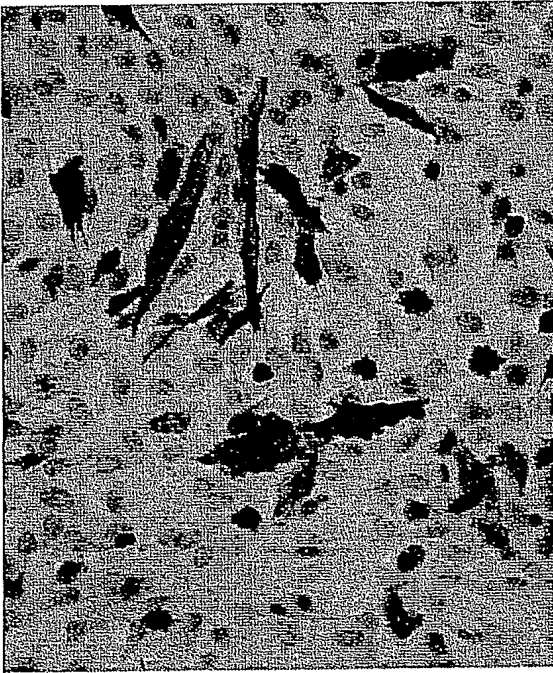
C2C12- Mouse Mighty Clone Proliferation Assay  
72 hrs Proliferation



**FIGURE 4**



Mighty over expressing  
C2 Myotubes



Control C2 Myotubes

FIGURE 5